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14. ABSTRACT The focus of this research is to discern the structural and biophysical features of small deoxyoligonucleotides that have significant biological properties including the inducement of Toll-like receptor 9 (TLR9) in the mediation of cellular invasion. Cell invasion (metastasis) is a significant problem in the control and treatment of breast cancer. Recent research from our laboratory has demonstrated enhanced cellular invasion in the MDA-MB-231 breast cancer cells by ODN-M362, a 25-base single-stranded CpG-containing deoxyoligonucleotide. Although the mechanism(s) for this induction is unknown, our studies reveal key insights into the structural and sequence requirements for DNA activation of this cellular invasion process. The deoxyoligonucleotides that are most effective in eliciting an invasion response have been shown to adopt stable structural motifs including stem-loops or hairpins or G-quadruplex structures. Sequence modifications have been designed to probe base sequence, structure, and stabilities that are required for initiating TLR-9 mediated cellular invasion. Our results demonstrate that these small deoxyoligonucleotides and the stability of their secondary structures play a pivotal role in eliciting the TLR9-induced invasion process.					
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Introduction:

The focus of this research is to correlate the influence of secondary structure and stability of short deoxyoligonucleotides on their ability to induce toll-like receptor 9 (TLR9) mediated cancer cell invasion. Cell invasion (metastasis) is a significant problem in the control and treatment of breast cancer. Our laboratory has previously demonstrated enhanced cellular invasion in the MDA-MB-231 breast cancer cells by CpG rich deoxyoligonucleotides such as ODN-M362, a 25-base single-stranded CpG and known agonist for TLR9. Although the mechanism(s) for this induction is unknown, our studies reveal a linkage between the structural and/or sequence requirements with DNA activation of the cellular invasion process. More importantly, recent studies from our laboratory have demonstrated that DNA fragments isolated from breast apoptotic cancer cells (cells treated with doxorubicin) were highly effective in eliciting TLR9-mediated cancer cell invasion. Unlike the TLR9 agonist, ODN-M362, these DNA fragments do not have the phosphorothioate backbone as required by ODN-M362 to be an effective TLR9 agonist. Our recent studies provide a direct correlation between structural stability that imparts nuclease resistance with induction of cell invasion. The two deoxyoligonucleotide structures that have been demonstrated to be most effective in TLR9 induced cell invasion are stem-loops or hairpins or G-quadruplex structures. Our studies have been focused on probing sequence modifications to discern influences on structure and stabilities and their correlation with TLR-9 mediated cellular invasion. Our studies demonstrate these small deoxyoligonucleotides to be highly effective in eliciting the TLR9-induced invasion process.

BODY

Statement of Work (Aim 3 – Graves) Characterization of the base sequence, secondary structure, and stabilities required for deoxyoligonucleotides for the induction of TLR9-mediated cellular invasion

Rationale: CpG deoxyoligonucleotides such as ODN-M362 has been known to exert significant cellular responses since reported by Krieg in 1995 (Nature 374, 546-549).(1) In 2001, Bauer and coworkers reported the linkage between ODN-M362 and hTLR9 through activation of a response to bacterial DNA in the cells innate immune response.(2) In 2008, apoptotic DNA was demonstrated to exert similar hTLR9 activation of cellular invasion.(3) Of fundamental interest to our research is the nature of the deoxyoligonucleotide-hTLR9 interaction. Specific Aim 3 will utilize a biophysical approach to directly measure these interactions with respect to synthetic DNAs designed to examine base sequence, secondary structure, and structural stabilities in their interactions with TLR9. High resolution NMR will be used to characterize the structural properties of many of these DNAs (hairpins) as well as their structural stabilities (along with DSC). Surface Plasmon Resonance (BiaCore) will be used in conjunction with ITC studies to determine the binding properties associated with hTLR9 constructs with both synthetic and native DNAs. These biophysical studies will be directly correlated with cell-invasion assays to determine the linkage between DNA properties and their abilities to modulate TLR9 induced cellular invasion.

Aim # 3: Probing the Role of DNA Sequence, Structure and Stability in TLR9 Mediated Induction of Cellular Invasion.

The mechanism(s) through which deoxyoligonucleotides may exert influence in TLR9 mediated biological responses remains unknown. The CpG deoxyoligonucleotide (ODN-M362) has long been demonstrated to influence TLR9-mediated biological activities; however, recent studies from this laboratory have shown that ODN-M362 to be highly influential in stimulating TLR9-mediated cellular invasion.(3) Our laboratory recently demonstrated comparable cellular invasion using DNA obtained from apoptotic cancer cells. This observation is highly significant since this DNA has a “native” sugar-phosphate backbone in contrast to the modified “phosphorothioated” backbone of the ODN-M362. Hence, the initial characterization of ODN-M362 as a TLR9 ligand may need to be expanded to more general terms of sequence, DNA secondary structure, and/or stabilities. A major focus of our research is to correlate these structural and biophysical properties of short nucleic acids with their effectiveness in modulating TLR9 mediated cellular activities. A better understanding of the nature of the interaction(s) between TLR9 and short deoxyoligonucleotides may provide key insights into strategies for circumventing activation of TLR9-mediated invasion processes.

Approach: The studies proposed in AIM 3 focus on examination and characterization of base sequence, secondary structural features, and stabilities of synthetic deoxyoligonucleotides. These deoxyoligonucleotides are designed to probe effects of changes in base sequence (CpG versus non-CpG), secondary structural properties (single-stranded, hairpin, duplex) and stabilities of concomitant secondary structures on interactions with TLR9, activation of TLR9-induced cellular invasion, and nuclease susceptibility. Our multifaceted approach will encompass a myriad of biophysical approaches including surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and differential scanning calorimetry (DSC) to gain insights into whether deoxyoligonucleotides of different sequences and/or structural motifs exhibit altered binding to TLR9. These studies will parallel cellular invasion assays to determine correlations between TLR9 activation by deoxyoligonucleotides with their induction of cellular invasion. From these studies using synthetic deoxyoligonucleotide systems we will expand our efforts to include apoptotic DNAs derived from breast cancer cells.

Task 3.1. Purification of TLR9. We have developed an inducible expression system for the portion of the extracellular domain of hTLR9 in HEK cells. This domain has a FLAG-his tag on the C-terminus portion. The hTLR9 will be purified by AKTA-FPLC System (GE Healthcare) with the final purification using a nickel column.

Task 3.2. Design and Characterization of Selected deoxyoligonucleotides to probe sequence, secondary structures, and stabilities as TLR9-mediating binding ligands. Deoxyoligonucleotides will be designed to determine the role of base sequence, structural, and stabilities as binding ligands for TLR9. Preliminary studies have demonstrated that the parent ODN-M362 (25-mer) can be reduced in size to a blunt-end hairpin (16-mer) with no loss in TLR9-mediated induction of cell-invasion. Further studies are underway to discern the structural and thermodynamic nature of the deoxynucleotide(s) and to determine the lower-limit in size that is needed for TLR9-activation. DSC and NMR (Bruker Avance II-700 with Cryoprobe) are used to characterize the secondary structures and stabilities associated with the deoxyoligonucleotides. Recent studies in our laboratory reveal secondary structure to play a critical role in TLR9 activation; however, this may be a secondary response due to the nuclease resistance imposed by secondary structural features, allowing the deoxyoligonucleotide to reach its intracellular target.

Task 3.3. SPR and ITC studies to determine whether DNA sequence, structure, or stability modulates TLR9 interactions. Current studies in our laboratory examining the role of deoxyoligonucleotides in inducing TLR9-mediated cellular invasion demonstrate a clear correlation; however, direct evidence of this interaction is required. With the ability to overexpress hTLR9 in inducible HEK cells, we now propose to directly examine the interactions of various DNAs (both demonstrated TLR9-ligands such as ODN-M362 as well as deoxyoligonucleotides that show no activity in the cellular invasion assay). From these studies, we hope to discern the structural and biophysical nature of the deoxyoligonucleotide-TLR9 interaction and gain insights as to how this interaction influences TLR9 mediated cellular invasion.

Progress to Date (July 15, 2011 – July 14, 2012)

Task 3.1. Purification of TLR9. In last year's annual report, I reported that TLR9 was available from a biotech company (BioClone, Inc. in San Diego, CA). Although they advertised hTLR9 was available, they could not deliver their product. We have now gone back to our original expression strategy. We have developed an inducible expression system for the portion of the extracellular domain of hTLR9 in HEK cells. This domain has a FLAG-his tag on the C-terminus portion. The hTLR9 will be purified by AKTA-FPLC System (GE Healthcare) with the final purification using a nickel column. The protein is pure and has been demonstrated quite stable for our biophysical studies which probe the preferential binding properties of selected DNA structural motifs and sequences.

Task 3.2. Design and characterization of selected deoxyoligonucleotides to probe sequence, secondary structures, and stabilities as TLR9-mediating binding ligands. We have made significant progress in designing novel deoxyoligonucleotides as short as 9 bases in length that can fold into a markedly stable hairpin structures. These deoxyoligonucleotides have demonstrated significant invasion inducing activities. The ability for this exogenous DNA to be added to a cell suspension, penetrate the cell membrane, and reach the TLR9 target, withstanding nuclease digestion, demonstrates that structural stability that induces

nuclease resistance and cellular persistence is important in the functioning of TLR9 agonists and broadens the biological role(s) of TLR9 to include much more than innate cellular immunity.

During this year, we have made significant progress in the characterization of several deoxyoligonucleotides that have been demonstrated by our laboratory to be highly effective in inducing TLR9-mediated cancer cell invasion. In contrast to ODN-M362 (type C CpG oligonucleotides that is recognized as a classic human TLR9 ligand) in which the sugar-phosphate linkages are phosphorothiolated to infer nuclease resistance, the deoxyoligo-nucleotides that we have found to be highly effective as TLR9-agonists in breast cancer cells have natural sugar-phosphate backbones and assume highly stable 3-dimensional structures resulting in nuclease resistance.

CpG ODN (ODN M362) is a known TLR9 agonist shown to stimulate innate cellular immune response

(ODN M362) 5'-d(TCGTCGTCGTTCTGAACGACGTTGAT)-3'
(contains phosphothioate backbone)

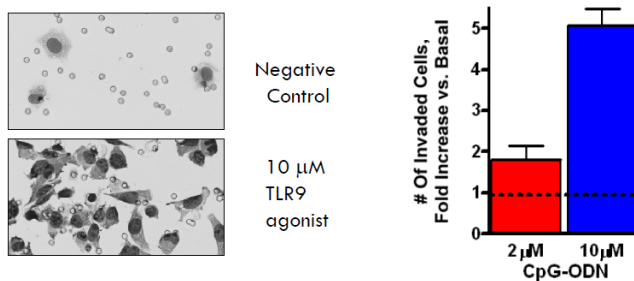


Figure 1. ODN-M362 is an effective TLR9 agonist and is shown to induce TLR9-mediated breast cancer cell invasion.

CpG rich deoxyoligonucleotides such as ODN-M362 have been known to exert significant cellular responses since reported by Krieg in 1995 (Nature 374, 546-549).⁽¹⁾ In 2001, Bauer and coworkers reported the linkage between ODN-M362 and hT LR9 through activation of a response to bacterial DNA in the cell's innate immune response.⁽²⁾ In 2008, apoptotic DNA was demonstrated to exert similar hTLR9 activation of cellular invasion.⁽³⁾ Of fundamental interest to our research is the nature of the deoxyoligo-nucleotide-hTLR9 interaction. Specific Aim 3 in this research will utilize biophysical approaches to probe

Our studies reveal ODN M362 induces TLR9-mediated breast cancer cell invasion.

the structural and energetic stabilities of deoxyoligonucleotides, their ability to modulate TLR9-induced cellular invasion, and finally, their correlations with TLR9 activation of the cell-invasion response. Over the past year, we have used high resolution NMR, differential scanning calorimetry, and isothermal titration calorimetry to probe the structural and energetic features of selected deoxyoligonucleotides. Upon successful expression and purification of hTLR9, we will use Surface Plasmon Resonance (BiaCore) in conjunction with ITC studies to determine the binding properties associated with hTLR9 constructs with both synthetic and native DNAs. These biophysical studies will be directly correlated with cell-invasion assays to determine the linkage between DNA properties and their abilities to modulate TLR9 induced cellular invasion.

Summary of work completed during this report period. Over the past year, we have focused our investigations on the role of stable secondary structures of deoxyoligonucleotides on their ability to induce toll-like receptor 9 (TLR9) mediated cancer cell invasion. Earlier studies from our laboratory (*Ilvesaro, et al (2008) "TLR9 mediates CpG-Oligonucleotide-Induced Cellular Invasion" Molecular Cancer Research 6, 1534-1543*) have demonstrated small deoxyoligonucleotides to have significant role in breast cancer metastasis (cell invasion) and may play an important role in the control and treatment of breast cancer.⁽³⁾ We started our investigations with the primary sequence of ODN-M362 (25-mer shown in Figure 1).

As a starting sequence we found that the ODN-M362 was a true TLR9 agonist only if the backbone was modified as a phosphorothioate. If the 25-mer sequence had a native backbone (normal sugar-phosphate), then the 25-mer was ineffective at eliciting TLR9-mediated cell invasion.

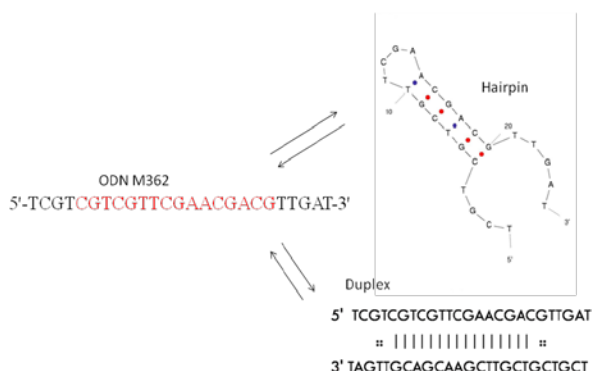


Figure 2. ODN-M362 sequence and several secondary structures that it can form; hairpin (top), duplex (bottom).

Hence, the comparison of the ODN-M362 with the apoptotic DNA fragments was ambiguous, since the fragmented DNAs would carry the “native” sugar-phosphate backbone rather than the phosphorothioate modification.

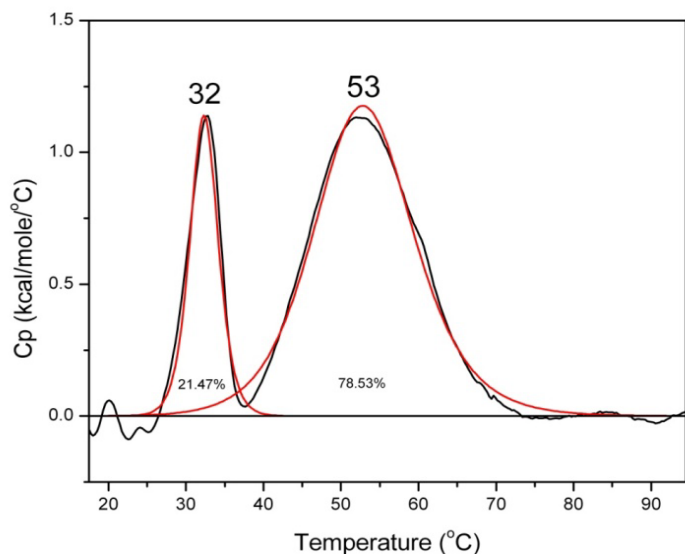
Working from this 25-mer sequence, we used molecular modeling, sequence analysis, and differential scanning calorimetry to determine potential secondary structures that could be formed from this sequence and came up with an ensemble of hairpins.

Table 1. Sequence variations of ODN M362 and other deoxyoligonucleotides.		
Name	Length	Sequence
ODN M362	25	5'-TCGTCGTCGTTCGAACGACGTTGAT-3'
Truncated	16	5'-CGTCGTTCGAACGACG-3'
5' end	20	5'-TCGTCGTCGTTCGAACGACG-3'
3' end	21	5'-CGTCGTTCTGAACGACGTTGAT-3'
Trunc + T	17	5'-CGTCGTTCTGAACGACG-3'
Trunc + TT	18	5'-CGTCGTTCTTGAACGACG-3'
9mer Hairpin	9	5'-CGCGAAGCG-3'
16mer purines	16	5'-CGTCGTGAAAACGACG-3'
h-Tel22	22	5'-TGGGTTAGGGTTAGGGTTAGGG-3'

The Table above shows several variations of the ODN-M362 sequence and through these sequences, our efforts to enhance both secondary structures and nuclease resistance.

Table 2. The melting temperature (T_m) of DNA hairpin structures in 100 mM NaCl BPES as determined by DSC.

Sequence	T_m (°C)
16mer (PS)	54.8 ± 0.53
17mer (PS)	58.15 ± 0.028
18mer (PS)	60.58 ± 0.038



The base sequence of the central region of ODN-M362 is self-complementary as illustrated in the bottom right of Figure 2. Analyses of the energetics of the central 16-mer where the 5' and/or 3' dangling ends are removed is shown in Table 2 and Figure 3. DSC analyses of this 16-mer (shown in Figure 3) revealed multiple structural species; with melting temperatures at 32 °C and 53 °C. This finding was quite surprising because of the self-complementarity of the 16-mer. Indeed, the predominant species was the hairpin species rather than the duplex species.

To further enhance the hairpin conformation, additional bases, T (17-mer) and TT (18-mer) were added in the center of the hairpin-loop region. As shown in Table 2, addition of T and TT to the loop region resulted in additional stabilization of the hairpin structure.(4-6) The fundamental question was whether these “thermally stable” DNA hairpins were effective in modulating TLR9-induced cell invasion.

Figure 3. DSC melting profiles of 100 μM truncated 16mer in Tris-EDTA buffer, pH 7.0.

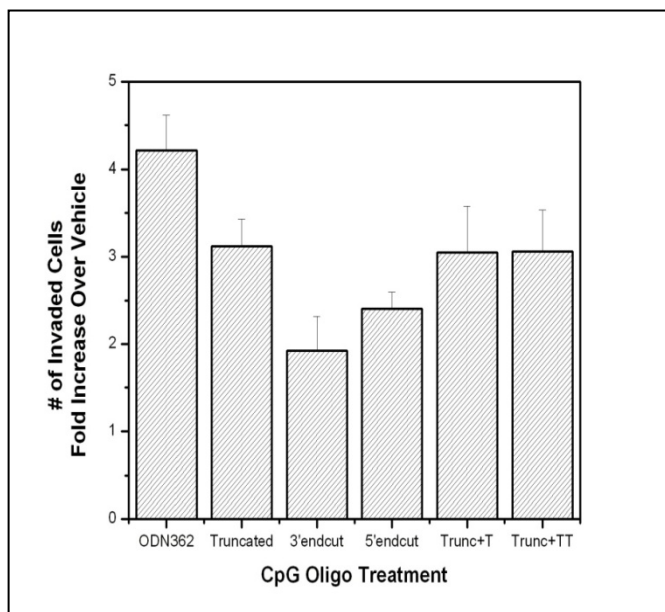


Figure 4. TLR9-induced cell invasion assay using selected hairpin-forming deoxyoligonucleotides.

These DNA hairpins were then tested for their ability to influence TLR9-mediated cancer cell invasion and as shown in Figure 4, the hairpins demonstrated considerable invasion-inducing capacity. Although the cell invasion level was not as high as the control ODN-M362, these DNA hairpins have a “native” sugar-phosphate backbone rather than the phosphorothioate backbone of the ODN-M362. Hence, these DNA hairpins fully mimic the DNA fragments released as apoptotic DNA.

The 9-mer Hairpin. A further refinement of the DNA hairpin model system arose by deleting bases from the stem (duplex portion of the hairpin) and mutating the base sequence within the hairpin loop region.

Modification of the loop sequences resulted in considerable diversity in hairpin stability. Table 3 (provided on the left) shows each of the 9-mer sequences and loop variations associated with these studies.

Table 3. Oligonucleotide sequences used in this study. All oligonucleotides were purchased from Midland Certified Reagent Company; Midland, Tx. The loop nucleotides are highlighted in red. G: guanine, C: cytosine, A: adenine, T: thymine, U: uracil, N: nebularine, and I: inosine.

Name	Sequence
9mer WT	d-CGCGAAGCG
Loop GTA	d-CGCGTAGCG
Loop GAT	d-CGCGATGCG
Loop GTT	d-CGCGTTGCG
Loop GUA	d-CGCGUAGCG
Loop GAU	d-CGCGAUGCG
Loop GUU	d-CGCGUUGCG
Loop IAA	d-CGCGAAGCG
Loop GAN	d-CGCGANGCG
Loop IAN	d-CGCGIANGCG

In 1994, Hirao reported a very stable hairpin containing a three-nucleotide (GAA) loop.(7) We incorporated this loop into our trimmed hairpin, removing the T and TT bases, resulting in the sequence, 5'-CGCGAAGCG-3'. A thorough biophysical characterization was done on this hairpin that demonstrated an unusually high melting temperature of 89 °C by differential scanning calorimetry (DSC). Structural analysis of our hairpin, 9-mer, by NMR reveals that this unusual stability results in part from Hoogsteen-base pairing between G4 and A6.(8,9) A more complete description of the structural and energetic features of this hairpin is provided below.

Thermal denaturation of mutated hairpins using differential scanning calorimetry (DSC), however, gave us an indirect means on not only demonstrating the existence of the reverse Hoogsteen pair, but also allowed us to quantify the stabilizing effects of key functional groups that exist in the loop. highly thermal stable with a melting temperature of 76 °C in 0.1M NaCl. The 9-mer hairpin was demonstrated to be nuclease resistant by UV-visible spectroscopy.(10)

Further analyses by NMR spectroscopy have revealed that G4 and A6 form a reverse Hoogsteen base pair. In order to demonstrate this relationship, structural NMR experiments and select nucleotide mutations of the loop bases were performed. Hirao was the first to solve a solution structure of a hairpin containing the GAA loop sequence, and although direct evidence of hydrogen bonding between his G3 and A5 was not apparent, he was able to point out key NMR characteristics of the hairpin, such as the upfield shifts for the non-hydrogen bonded G3 imino and the A4 4' sugar residue. Proton NMR experiments performed by James and coworkers was also unable to demonstrate the reverse Hoogsteen base pair directly, but did note a strong NOE cross peak between the loop guanine H1' and the second loop adenine H8.(11)

Purified synthetic oligonucleotides referenced in Table 3 were purchased from Midland Certified Reagent Company; Midland, TX and dissolved in BPES buffer (10mM sodium phosphate buffer with 1mM EDTA pH 7.0) containing 100mM NaCl. The deoxyoligonucleotide was then annealed by heating the sample above the melting point and then slowly cooling to room temperature. Sample concentrations were determined spectroscopically at 260nm on a Cary 100 UV-vis spectrophotometer at 90 °C. To probe the effects of reverse-Hoogsteen base pairing, a series of mutations at the 4, 5, and 6 base positions were undertaken as described in Table 3. Mutations of the bases within the loop sequence were chosen so as to perturb either loop base stacking and/or hydrogen bonding between G4 and A6. Mutating the G4 to an inosine or A6 to nebularine, results in the loss of the reverse-Hoogsteen base pairing capability either separately or together while maintaining optimal base stacking conditions. Mutations of 5-position base from an adenine to thymine or uracil allowed us to analyze disruptions in base stacking within the loop sequence.

Differential Scanning Calorimetry - The melting temperatures and the associated heats of unfolding for each hairpin were determined using a Microcal VP-DSC (GE Healthcare, Northampton, MA). Deoxyoligonucleotide samples were prepared at strand concentrations from 200 – 400 μ M in BPES buffer and verified by UV-Vis spectroscopy. Each sample was melted at a rate of 90 °C/hour from 5-120 °C and repeated no less than 5 times. Reference scans were also produced by analyzing buffer versus buffer in the same manner as the samples. Data were analyzed using Origin 7.0 VP-DSC software by first subtracting a reference scan (buffer versus buffer) from the raw data and the normalizing to strand concentration, producing heat capacity versus temperature plots. The data was then baseline corrected by connecting the pre and post-transition baselines with the cubic function provided within the software and subtracting the resulting baseline from the data. From the thermodynamic relationship, $\Delta H_{cal} = \int \Delta C_p(T) dT$, integrating the total area under the resultant melting curve provides the enthalpy of unfolding (ΔH_{unfold}), while the midpoint of the transition provides the melting temperature (T_m). The change in Gibb's free energy (ΔG) can then be derived by using $\Delta G^0(T) = \Delta H[1 - \frac{T}{T_m}]$ at any reference temperature, for our calculations the Gibb's free energy was calculated at 37 °C.

Table 4. DSC analysis of 9mer WT and loop mutants. Each sample was analyzed at known concentrations between 200 – 400 μ M. The samples were melted from 5 – 120 °C at a rate of 90°C/hour.

Loop	T_m (+/- °C)	ΔT_m (°C)	ΔH (+/- kcal/mol)	$\Delta \Delta H$ (kcal/mol)	$\Delta G_{37^\circ C}$ (+/- kcal/mol)	$\Delta \Delta G_{37^\circ C}$ (kcal/mol)
WT (GAA)	88.46 (0.06)	NA	19.08 (0.39)	NA	13.39 (0.28)	NA
GTA	84.09 (0.51)	-4.37	16.59 (0.44)	-2.49	11.66 (0.32)	-2.03
GAT	63.30 (0.20)	-25.16	7.88 (0.38)	-11.20	4.77 (0.22)	-8.92
GTT	65.42 (0.49)	-23.04	8.38 (0.39)	-10.70	5.18 (0.23)	-8.51
GUA	84.61 (0.48)	-3.85	16.82 (0.37)	-2.26	11.85 (0.25)	-1.84
GAU	62.06 (0.09)	-26.40	7.54 (0.41)	-11.54	4.50 (0.25)	-9.18
GUU	66.07 (0.23)	-22.39	8.23 (0.42)	-10.85	5.12 (0.26)	-8.57
IAA	69.10 (0.38)	-19.36	15.49 (0.59)	-3.59	9.89 (0.37)	-3.80
GAN	77.31 (0.62)	-11.14	17.62 (0.24)	-1.46	11.92 (0.12)	-1.76
IAN	64.73 (0.49)	-23.73	11.06 (0.45)	-8.02	6.79 (0.28)	-6.90

Differential scanning calorimetry allows us to monitor the change in heat capacity at constant pressure while increasing temperature. From this, we can calculate the melting temperature, the change in enthalpy upon melting and the standard Gibbs free energy at a given temperature. As seen in Table 4, mutations within the loop sequence have significant influence on the thermodynamic stability of the 9-mer hairpin. By mutating A₆ to a thymine, we see a decrease of 25 degrees the melting temperature, while a mutation of A₅ to thymine only decreases the T_m by 4 degrees. In contrast, if we analyze the changes in enthalpy we see that mutating either adenine to a thymine has a destabilizing effect. The changes in melting temperature may be attributed to a

disruption in both hydrogen bonds between G₄ and A₆, as well as a disruption in stabilization of base stacking contributed by both adenines.

In order to determine the magnitude of the contribution of the reverse Hoogstein base pair to the native structure without disrupting the stabilizing base stacking effects, we selectively mutated G₄ to an inosine base residue and A₆ to nebularine base residue.⁽⁹⁾ As seen in Figure 4, both inosine and nebularine resemble their parent structures of guanine and adenine perfectly except for the absence of their respective amino groups.

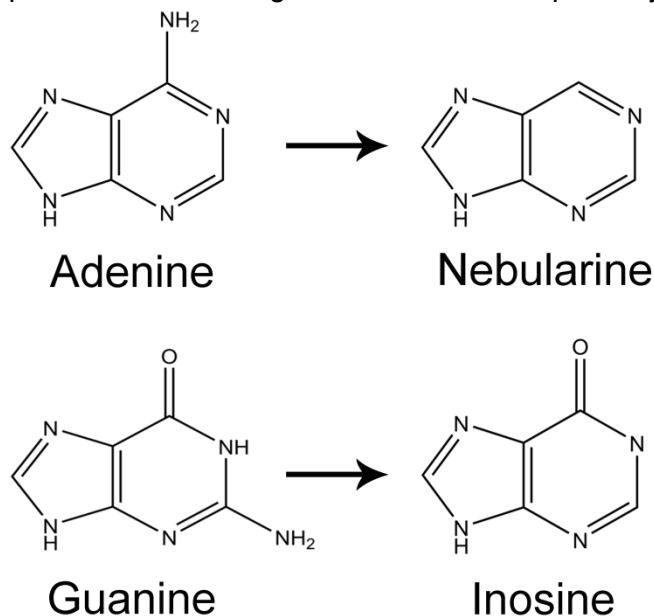


Figure 4. Mutations of adenine to nebularine or guanine to inosine allows us to selectively remove hydrogen bond donating groups without perturbing the base stacking contributions of the native base.

As seen in Table 4, these mutations also decrease the melting temperature, enthalpy and free energy. Mutating G₄ to inosine drops the melting temperature by 19 degrees. Mutating the A₆ to nebularine results in an 11 degree decrease in the T_m. When both the G₄ and A₆ are mutated so that the ability to form the reverse Hoogstein base pairing is lost, the melting temperature drops by 24 degrees, similar in magnitude to that of mutating A₆ to thymine; however, the enthalpy of melting is slightly higher for loop IAN than for GAT, suggesting that the thermal stability of the loop is largely dependent on the base pairing of G₄ and A₆ as well as the base stacking interactions within nucleotides between the loop and stem.

It is also interesting to note that by removing the hydrogen bond donor capabilities of adenine by mutating to nebularine, we do not see the same effect on the melting temperature as we do when we mutate the guanine. It is possible to imagine that the guanine hydrogen bond is more stabilizing than that of the adenine's should the guanine base be tilted along the phosphate backbone in such a way as to not disrupt the guanine's hydrogen bond but to make adenine's more flexible and thus less stable. This degree of tilt could be attributed to the stacking of A₅ with its amino group juxtaposed over G₄, constricting G₄ between C₃ and A₅ as seen in the solution structure in figure #. The stacking of A₅ upon G₄ instead of upon A₆ also allows A₆ a slightly higher freedom of rotation about the phosphate backbone, which could also destabilize its hydrogen bond to guanine. Regardless, it is interesting to note that within the 9-mer hairpin, not all hydrogen bonds are equal.

In addition, we examined the effects of mutating adenine to either thymine or uracil. Both T and U seemed to have the same effect on the stability of the hairpin. Mutating A₅ to either thymine or uracil appears to result in only slight decrease the loop stability; in contrast, mutating A₆ dramatically decreases the thermal stability. It was initially postulated that a mutation to a thymine residue would result in a larger effect on the loop stability when compared with uracil due to the presence of the bulky hydrophobic methyl group; however our studies suggest that there are no significant energetic differences between the T and U mutations. This may suggest that the methyl groups are buried within the molecule as opposed to being exposed in the solvent as suggested by from molecular modeling studies. Additional studies are underway to determine the hydration properties of the 9-mer mutants to discern the role of bound waters on structural stability.

Structural Analysis of the 9-mer Hairpin - Samples for NMR analysis were prepared at single strand concentrations of 1mM in BPES buffer. For analysis of exchangeable protons, samples were prepared in H₂O buffer and 10% D₂O was then added. For NMR analysis in 100% D₂O, the water samples were lyophilized and reconstituted into the same initial volume with D₂O, lyophilization and reconstitution in D₂O was repeated three times to remove trace amounts of H₂O. All of the NMR experiments were performed on a Bruker Ultrashield 700 MHz spectrophotometer coupled with a Bruker TCI cyroprobe. NOESY spectra for 90% water and 100% D₂O samples were obtained at various mixing times ranging from 50 to 400 ms at 298K. COSY, DQFCOSY and TOCSY spectra were also obtained at 298 K to determine scalar coupled protons. Solvent suppression was achieved using the Bruker derived excitation sculpting gradient pulse (find source). All spectra were then processed using Bruker Topspin 2.1.

NOE peaks were assigned and gaussian fit integrated using SPARKY (Goddard). The peaks from each NOESY spectra were used for distant constraints while all other spectra were used to confirm peak assignments. Distances derived from non-overlapping peaks were assigned an error of +/- 10% while overlapping peaks were given an error of up to +/- 50%. The SPARKY derived integrated peaks from each spectra were then averaged, normalized and converted to distance constraints using RANDMARDIGRAS (Liu, 1995) from MARDIGRAS (Borgias, 1990). The resulting distance restraints were then converted for use with AMBER by MARDIGRAS. The solution structure was then derived by coupling the resulting NMR distance restraints with a molecular dynamic simulated annealing procedure using generalized Born implicit solvation with AMBER utilizing the previously derived theoretical structure.

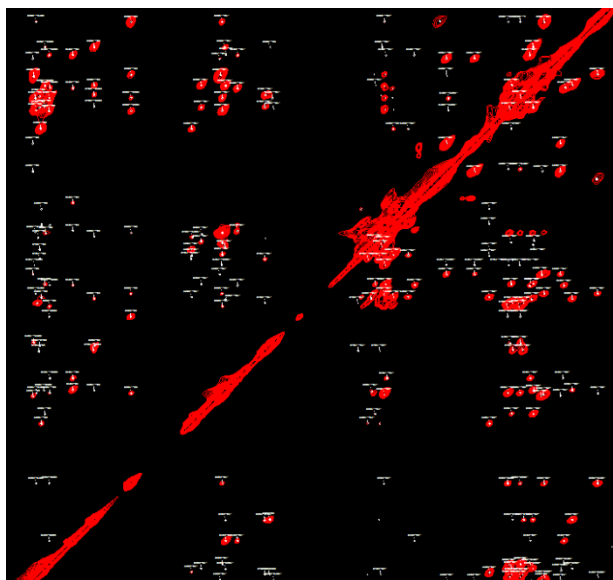


Figure 5. Representative NOESY data obtained for the 9-mer deoxyoligonucleotide hairpin, 5'-CGCGAAGCG-3'. Data were obtained on a Bruker Avance II 700 MHz NMR with TCI Cryoprobe at 25 °C. A mixing time of 250 ms was used.

NMR melts of samples in 100% D₂O BPES were performed on a Bruker ultrashield 400MHz with BBO probe. 1D proton NMR spectra were collected from a temperature range of 25 to 90 °C at intervals of 5 °C (data not shown). Spectra were processed and analyzed using Bruker Topspin2.1. With DSC analysis, we were able to monitor the temperature at which the 9mer hairpin melted, but with NMR analysis, we were able to monitor the sequential unfolding of the hairpin. A plot of the change in the chemical shift ($\Delta\delta$) of the aromatic and imino protons with increasing temperature allows us to determine the melting temperature of each base pair and discern a mechanism for the melting of the hairpin. A plot of the shift changes versus temperature provides a hyperbolic curve,

where the maximum of the curve indicated the temperature at which the base the aromatic proton belongs to is no longer paired with its partner.

Table 3. NMR peak assignments of 9-mer WT. Peaks were assigned based on NMR NOESY, COSY, DQFCOSY and TOCSY spectra collected on a 1 mM sample of 9-mer WT in 90:10 H₂O:D₂O BPES buffer and 100% D₂O BPES buffer at 298 K on a Bruker 700 MHz NMR with TCI cryoprobe. Shifts are reported in ppm.

	H1'	H2'/H2''	H3'	H4'	H5'/H5''	H1	H2/H8	H5/H6
C1	5.683	1.944/2.333	4.598	3.968	3.656			5.817/7.556
G2	5.843	2.585/2.443	4.841	4.245	3.917	12.96	7.833	
C3	5.858	1.561/2.170	4.705	4.378	4.005			4.974/6.901
G4	5.271	2.447/2.573	4.82	4.262	3.731/3.910	10.54	7.995	
A5	5.884	2.195	4.492	1.985	3.29/2.969		7.990/8.017	
A6	6.207	2.798	4.745	4.19	3.907/3.732		7.909/7.931	
G7	5.367	2.406/2.535	4.813	4.308	4.113/4.058	12.99	7.956	
C8	5.82	1.853/2.302	4.765	4.162	4.038			5.324/7.316
G9	6.096	2.295/2.552	4.600	4.117	4.008		7.885	

NMR solution structure

High resolution NMR provides a unique method for determining the structural features of biomolecules in their hydrated state.(12,13) This is especially important for nucleic acids whose structures and stabilities are highly dependent on their hydration state. The solution structure for the native 9-mer hairpin was determined by

normalizing and averaging the assigned NOE peaks (Table 3) from three NOESY experiments with mixing times ranging from 50 to 300 ms. The peak areas were converted to distance constraints and used by AMBER to derive the solution structure as seen in Figure 6. The NMR structure for the 9-mer hairpin (5'-CGCGAAGCG-3') is in good agreement with the 6-mer hairpin (Hirao 1994).(8) Our NMR studies reveal persistent exchangeable resonances assigned to the G4 2-amino and A6 6-amino resonances. The hairpin structure derived from AMBER put these protons within 1.8 Å of their respective H-bond acceptor atoms (N7 on A6 and N3 on G4) making this reverse Hoogsteen base pair highly probable for the G4 and A6 bases. Additional support for reverse Hoogsteen basepairing comes from our observation of NOE crosspeaks between H4' of the G₄ sugar to the H2 of A₆. The AMBER structure predicts the distance between these protons to be 4.2 Å, well within distance for observation of NOE contacts. The majority of the sugar residues were readily assigned and agree well with previous reported assignments for similar structures (Hirao, Ulyanov), especially with signature shifts such as the upfield shifts of A5 H4' around 2 ppm and the upfield shift of the nonhydrogen bonded G4 H1 around 10 ppm that disappears at temperatures above 10 °C.

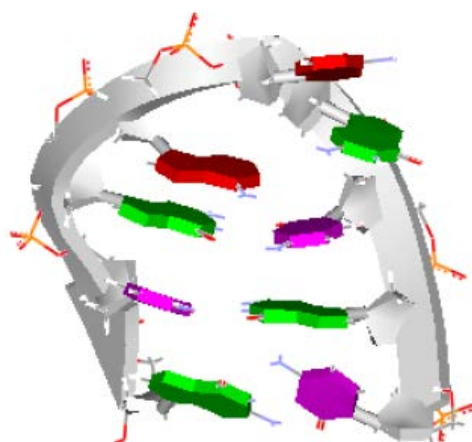
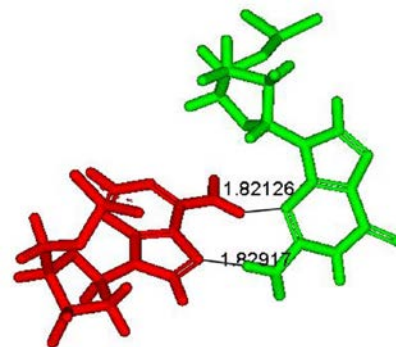


Figure 6. (Left) Solution structure the 5'-dCGCGAAGCG-3' hairpin derived from NOE distance constraints from NOESY and TOCSY NMR data and AMBER.

The NMR solution structure of the 9-mer hairpin is shown in Figure 6. NMR studies reveal the presence of the persistent exchangeable resonances (G4 2-amino and A6 6-amino) formed by the reverse Hoogsteen base pairs formed between G4 and A6 providing direct evidence of loop stabilization for the hairpin.

Figure 7. (Right) The reverse Hoogsteen base pairing formed between the G4 2-amino and A6 6-amino protons are illustrated.



Energetic evidence provided by both the by DSC analysis of loop mutations as well as the NMR melts supports the GAA tri-loop structure. When the hydrogen bond donor capabilities of adenine is eliminated by mutating A6 to the modified base, nebularine, we do not see the same effect on the melting temperature as we do when we mutate G4 to inosine.

Although the formation of the reverse Hoogsteen base pair is evident from the NMR structure, the marked stability of the 9-mer hairpin cannot be explained solely because of these two H-bonds stabilizing the G-A base pair. At best, the reverse Hoogsteen base pair may contribute 4-5 kcal/mol of additional stability to the hairpin. However, we speculate that the guanine provides a more stabilizing effect than adenine due to the orientation of the guanine due to a more favorable stacking conformation. The degree of tilt of this base may be attributed to the stacking of A5 with its amino group juxtaposed over G4, constricting G4 between C3 and A5 as observed in the solution structure. Furthermore, the stacking of A5 upon G4 instead of upon A6 also allows A6 greater flexibility about the phosphate backbone, which could have destabilizing effects with its hydrogen bond to guanine.

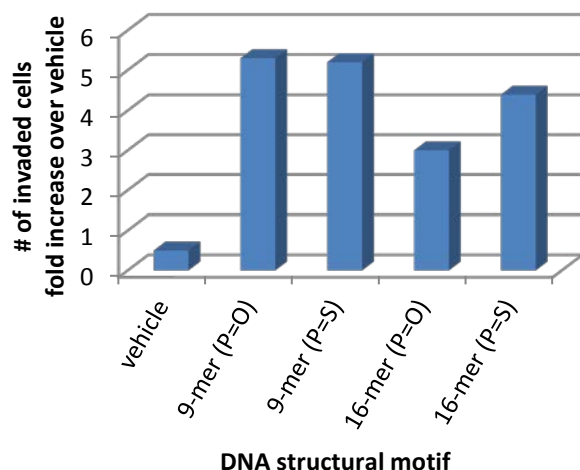


Figure 8. TLR9-induced cell invasion assay using selected hairpin-forming deoxyoligonucleotides. The 9-mer hairpin (native backbone and phosphorothioate backbone) show no difference in their stimulation of TLR9 mediated cell invasion, indicating the 9-mer (native backbone) is just as nuclease resistant as the modified backbone.

This difference in stabilization between the G4 and A6 is also reflected in the NMR melting studies. Analyses of the shift changes upon heating of the G4 and A5 aromatic protons (H8 and H2) reveal little changes until the temperature reaches 60 °C, whereas the aromatic protons of A6 (H8 and H2) show chemical shift changes at much lower temperatures, indicative of greater flexibility of A6 as compared to G4 and A5. The stability of the 9-mer hairpin structure results in this deoxyoligonucleotide being resistant to nuclease digestion (data not shown). However, the true test of our hypothesis was to determine whether this 9-mer hairpin would be effective in eliciting a TLR9-mediated cell invasion response. Figure 8 shows the results of our invasion assay studies, clearly indicating that the 9-mer was highly effective (even as a “native” sugar-phosphate backbone) in inducing TLR9-mediated cell invasion.

A summary of our findings with the 9-mer hairpin is provided below:

- The 9-mer d(C₁G₂C₃G₄A₅A₆G₇C₈G₉) is very proficient in inducing TLR9-mediated cancer cell invasion.
- This 9-mer does not require phosphothioate backbone modification to be effective in matrigel invasion assays; the phosphodiester (native DNA sugar-phosphate linkage) is sufficient.
- The 9-mer form an unusually stable hairpin structure, with a T_m of 88 °C.
- The structure of this 9-mer hairpin as determined by NMR shows increased stabilization due to the G₄A₅A₆ loop sequence wherein the G₄ and A₆ form a Hoogsteen-like base pair and the A₅ stacks across this G₄ : A₆ base pair.
- The unusual stability of this GAA tri-nucleotide loop is now being implemented in other sequences to provide a “molecular staple”.

The Human Telomeric G-quadruplex as a TLR9 Agonist. Our laboratory is exploring the design, synthesis and characterization of a number of other structurally stable DNAs that due to their structural stability are rendered nuclease resistant and have persistence within the cell. Of particular interest to us is the human telomeric sequence d[AGGG(TTAGGG)]₃. This sequence is found at the ends of human chromosomes and due to the nature of the repetitive GGGs forms G-tetrads that consequently stabilize into a stable secondary structure known as the G-quadruplex (Figure 10). (14) Of particular interest to this project is quest to characterize the properties and/or features of the “apoptotic” DNA that can induce TLR9-mediated cancer cell invasion, the endogenous human telomere DNA may be of significant interest. Breast cancer cells up-regulate telomerase resulting in the continuous elongation of the telomere region of the chromosomes; hence imparting immortality to the cancer cells. Upon treatment of cancer cells by chemotherapeutic agents that induce cellular apoptosis, the DNA in the cells are degraded by a host of nucleases. The surviving DNA fragments from the apoptotic cells must have some mechanism for nuclease resistance.

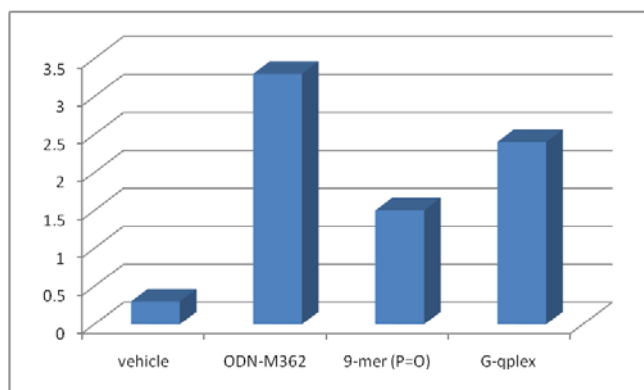
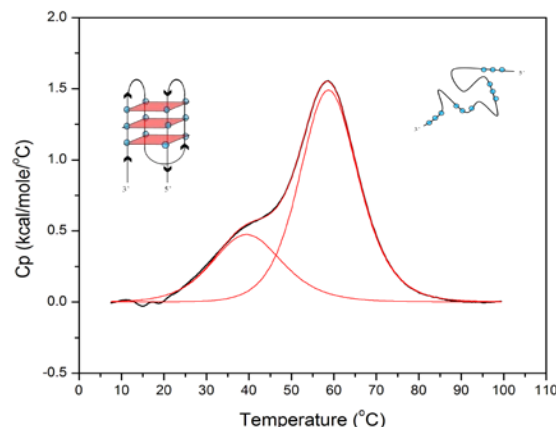


Figure 9. Parental MDA-MB-231 cells treated with 5 μM (strand) ODN-M362, 9-mer (wild type) or hTel-22 G-quadruplex in Matrigel-matrix. Induction of breast cancer cell invasion by deoxyoligonucleotides mediating TLR9. The human G-quadruplex (h-Tel 22 mer) is highly efficient at stimulating TLR9 mediated cell invasion.

The human telomeric DNA consists of a repetitive sequence that is approximately 150-200 kilobases in length. (14) The terminal end of this sequence has a 3' single stranded overhang with the tandem repeat of 5'-TTAGGG-3' that has been demonstrated to form stable G-quadruplex structures, *in vitro*. (15,16) The formation of G-quadruplex structure inhibits the attachment of telomerase and the enzymatic extension of the telomere that is often associated with most cancer cells. (17, 18) The human telomeric G-quadruplex structure has three stacked G-tetrads connected by -TTA- loops. The nature of the loop connectivity gives rise to the alternate conformations that have been observed by NMR and X-ray crystallographic methods under varying solution conditions. The most notable structural differences are between the sodium and potassium forms. Sodium based buffers result in an all antiparallel strand orientation with glycosidic bond angles that are all anti conformations. However, the loop connectivity may differ and

either be all lateral loops, the “chair” conformation or have two lateral loops and one diagonal loop, the “basket” conformation, both Na⁺ conformations and their loop connectivity can be seen in Figure 10.

Figure 10. DSC experiment showing the thermal melting transition of G-quadruplex structure formed by hTel-22 in sodium phosphate buffer.



The energetics of G-quadruplex unfolding and the enthalpic (ΔH_{unfold}) and entropic (ΔS_{unfold}) contributions to the thermal stability have been reported by several research groups using a variety of methods. Shown in Figure 10 is a DSC analysis of h-Tel 22 G-quadruplex stability in Na⁺ buffer. Our data shows this G-quadruplex structure to melt at approximately 60 °C and undergo two transitions. In other data (not shown, we have monitored the G-quadruplex structure by CD spectroscopy, showing characteristic positive peak at 295 nm. As described in Figure 6, we have monitored the hTel-22 G-quadruplex DNA in the presence of a variety of nucleases and found this structural motif to infer nuclease resistance to the DNA. We have also examined the ability of this DNA to induce TLR9-mediated cancer cell invasion.

To further investigate sequence effects on the structural stability of the h-Tel G-quadruplex motif, we have investigated the effects of changes to the 5'-flanking end of the h-Tel 22-mer. These changes and the resulting energetic profiles for G-quadruplex unfolding are provided in Table 4 (below).

Table 4. DSC results for 5' modifications to the h-Tel G-quadruplex.

Name	Sequence	ΔH (kcal/mol)	T _m (°C)
h-Tel 22mer	5'-AGGG(TTAGGG) ₃ -3'	37.8	67.2
h-Tel 23mer	5'-TAGGG(TTAGGG) ₃ -3'	43.6	65.4
h-Tel 24mer	5'-TTAGGG(TTAGGG) ₃ -3'	51.2	62.4

Addition of a T to the 5' end of the h-Tel 22 results in no significant change in the unfolding temperature (2 degree drop), but a significant increase in the unfolding energy. Addition of a second T to form the h-Tel 24mer continued in this trend, further decreasing the unfolding temperature, but significantly increasing the unfolding energy (13.3 kcal/mol) compared with the h-Tel 22mer. We are currently working to see if these energetic changes can be correlated with the ability of these telomeric sequences to modulate TLR9-mediated cancer cell invasion.

Task 3.3. SPR and ITC studies to determine whether DNA sequence, structure, or stability modulates TLR9 interactions. The focus of this specific aim in year 2 has been to examine the interactions of the selected deoxyoligonucleotides described in Task 3.2 as well as others that are currently under examination, with TLR9 using a variety of biophysical methods. Because of our difficulty in acquiring TLR9, this aim has not been completed. However, we have received DOD-BCRP approval for a no-cost extension so that the TLR9 binding studies can be completed. With our new personnel on board, we will be able to express and purify human TLR9 in quantities sufficient to carry out the proposed Task 3.3 studies.

KEY RESEARCH ACCOMPLISHMENTS OVER THE PAST YEAR:

- Completion of the deoxyoligonucleotides design and characterization based on the ODN-M362 sequence.
- Development and characterization of an unusually stable 9-mer hairpin [d(C₁G₂C₃G₄A₅A₆G₇C₈G₉)] that is stable to 89 °C.
- Demonstrated that this 9-mer hairpin does not require phosphorothioate backbone modification to be nuclease resistant.
- Demonstrated this 9-mer to be highly efficient at inducing TLR9-mediated cancer cell invasion.

- Completed thermodynamic studies on 9-mer hairpin and loop mutations to discern the presence of a reverse-Hoogsteen base pairing.
- Completed NMR studies to discern the solution structure of the 9-mer [d(C₁G₂C₃G₄A₅A₆G₇C₈G₉)] hairpin.
- Demonstrated that the h-Tel22 G-quadruplex is nuclease resistant in the folded conformation.
- Demonstrated the h-Tel22 G-quadruplex DNA formed from the human telomeric repeat sequence is highly effective as a TLR9 agonist in inducing cancer cell invasion.
- Demonstrated addition of bases to the 5'-end of the h-Tel 22mer resulted in significant changes to the folding energy of the G-quadruplex motif.

REPORTABLE OUTCOMES:

- Manuscripts Published
 - Ketron, A.C., Denny, W.A., Graves, D.E. and Osheroff, N. (2012) "Amsacrine as a Topoisomerase II Poison: Importance of Drug-DNA Interactions" *Biochemistry* 51, 1730-1739.
 - Aldred, K.J., McPherson, S.A., Wang, P., Kerns, R.J., Graves, D.E., Turnbough, C.L. and Osheroff, N. (2012) "Drug Interactions with Bacillus anthracis Topoisomerase IV: Biochemical Basis for Quinolone Action and Resistance" *Biochemistry*, 51(2) 370-381.
 - Pitts, S., Jablonsky, M., Duca, M., Dauzonne, D., Monneret, C., Arimondo, P., Anklin, C., Graves, D.E., and Osheroff, N. (2011) "Contributions of the D-Ring to the Activity of Etoposide Against Human Topoisomerase II: Potential Interactions with DNA in the Ternary Enzyme-Drug-DNA Complex" *Biochemistry*, 50(22) 5058-5066.
 - Gentry, A.C., Pitts, S.L., Jablonsky, M.J., Bailly, C., Graves, D.E., and Osheroff, N. (2011) "Interactions between the Etoposide Derivative F14512 and Human Type II Topoisomerases: Implications for the C4 Spermine Moiety in Promoting Enzyme-Mediated DNA Cleavage". *Biochemistry*, 50(15), 3240-3249.
- Manuscripts currently in preparation:
 - Hudson, J.S., Ding, L., Ma, J. Lewis, E. and Graves, D.E. (2012) "Recognition and Binding of Human Telomeric G-Quadruplex DNA by Unfolding Protein 1 (UP1)", manuscript in preparation for *Biochemistry*.
 - Kauppila, J.H., Karttunen, T.J., Saarnio, J., Nyberg, P., Salo, T., Graves, D.E., Lehenkari, P.P, and Selander, K. (2012) Short DNA Sequences and Bacterial DNA Induce Invasion in Gastrointestinal Malignancies *in vitro*, *manuscript submitted to Cancer Research*
 - Hayden, K.L., and Graves, D.E. (2012) "Structural and Thermodynamic Characterization of a Highly Stable DNA Hairpin", manuscript in preparation for *Nucleic Acids Research*.
 - Hudson, J.S., Ding, L., Lewis, E., Graves, D.E. (2011) "Quadruplex Unfolding: Influence of Loop Mutations on Structural Stability", manuscript in preparation for *Biochemistry*.
- Abstracts from presentations at regional and national meetings:
 - Lanier, Kate and Graves, David E. Structural and thermodynamic analysis of a highly stable DNA hairpin. 63rd Southeast Regional Meeting of the American Chemical Society, Richmond, VA October 26-29, 2011.

- Aldred, Katie J., McPherson, Sylvia A., Lindsey, R. H.; Wang, Pengfei; Kerns, Robert J., Graves, David E., Turnbough, Charles L. and Osheroff, Neil. "Quinolone Resistance in *Bacillus anthracis* Type II Topoisomerases" 63rd Southeast Regional Meeting of the American Chemical Society, Richmond, VA October 26-29, 2011.
 - Brooks, S., Selander, K.S., Harris, K.W., and Graves, D.E. (2011) "Structure and Stability of Deoxyoligonucleotides that Induce TLR9-mediated Cancer Cell Invasion". Era of Hope – Congressionally Directed Medical Research Programs, Orlando, FL, August 2-5, 2011.
 - Lanier, K.L., Ottenfeld, E. and Graves, D.E. (2010) "Structural Analysis of a Highly Stable DNA Hairpin". Joint 66th Southwest and 62nd Southeast Regional Meeting of the American Chemical Society, New Orleans, LA, Dec. 1-4, 2010.
- Degrees Awarded
 - none

CONCLUSION: The research that has been completed over the past year continues to demonstrate a direct correlation between structural stability of deoxyribonucleotides and their ability to induce TLR9-mediated cancer cell invasion. Our research focused on the structural and biophysical characterization of two key stable DNA structures; the 9-mer hairpin formed by 5'-CGCGAAGCG-3' and the human telomeric G-quadruplex formed by 5'-AGGG(TTAGGG)₃-3'. Both structural motifs were demonstrated to be nuclease resistant; hence, mimic DNA fragments released upon cellular apoptosis rather than requiring backbone modifications as needed for ODN-M362. We have utilized calorimetry (ITC and DSC) to characterize the structural stability and high resolution NMR to determine the solution structure of the 9-mer hairpin. We found an unusual reverse-Hoogsteen base pair between the G4 and A6 in the tri-base loop and that this interaction was needed to fully stabilize the hairpin. Although the two hydrogen bonds generated by the G4.A6 interaction are not sufficient to explain this markedly high stability, their presence is speculated to be needed to direct proper base stacking at the interface of the loop and stem regions.

We are very excited with our findings that the human telomeric G-quadruplex can induce TLR9-mediated cancer cell invasion. This is exciting for two reasons: First, this is an endogenous sequence found to be overexpressed in all cancer cells due to the upregulation of telomerase. The extension of the telomeric region of the chromosomes results in unlimited capacity for cell division; hence, cancer cell immortality. For this reason, the telomere and telomerase have been a novel target for future anticancer drug development. Secondly, the human telomere can fold into discrete highly compacted 3-dimensional structures known as the G-quadruplex that we have shown to be nuclease resistant. Hence, in cancer cells and in particular, cancer cells treated with chemotherapeutic agents and induced to undergo apoptosis, the DNA is digested and released from the dead cell. DNA surviving this apoptotic process must be nuclease resistant.

On the dark side, it appears that human telomere G-quadruplex DNA is highly efficient in induction of TLR9-mediated cell invasion pathway. Hence, it is of critical importance to discern the role of these structurally stable, nuclease resistant DNAs in their ability to modulate TLR9-mediated pathways.

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APPENDICES:

Curriculum Vitae

SUPPORTING DATA:

NA

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PERSONAL STATEMENT

Dr. David Graves has extensive experience with biophysical approaches examining the structural and functional properties of nucleic acids, nucleic acid-ligand interactions, and protein-nucleic acid interactions. He uses microcalorimetry (ITC and DSC) to discern thermodynamic properties of these interactions and structural stabilities. NMR is the primary method used for structure determination of biological macromolecules; specifically nucleic acid-ligand interactions. His research also focuses in areas of drug-discovery, with emphasis in anticancer and antiviral drugs as well as the role of small DNAs as TLR ligands in stimulating biological responses such as innate cellular immunity and cancer metastasis. Dr. Graves is a senior research scientist in the UAB Comprehensive Cancer Center, the Center for Nanoscale Materials and Biointegration, Center for Lung Health, Center for Biophysical Sciences and Engineering, Center for Structural Biology, and the Center for Information Assurance and Joint Forensics Research and holds a secondary faculty appointment in the UAB Department of Biochemistry and Molecular Genetics.

EDUCATION:

1970-1974	University of Alabama at Birmingham, B.S. (1974), Chemistry
1974-1979	University of Alabama at Birmingham, Ph.D. (1979), (Biochemistry & Molecular Biology in Laboratory of Dr. K. L. Yielding)
1980-1984	Postdoctoral - Department of Chemistry, University of Rochester, Rochester, New York. (Biophysical Chemistry in laboratory of Dr. Thomas R. Krugh)

RESEARCH AND PROFESSIONAL APPOINTMENTS:

1974-1976	Graduate Research Assistant in Laboratory of Reproductive Biology, University of Alabama at Birmingham School of Medicine
1976-1979	Graduate Research Assistant in Laboratory of Molecular Biology, University of Alabama at Birmingham
1980-1984	Postdoctoral Fellow in Chemistry, University of Rochester
1984-1990	Assistant Professor of Chemistry, University of Mississippi
1990-1996	Associate Professor of Chemistry, University of Mississippi
1996-2003	Professor of Chemistry, University of Mississippi
1996-2003	Director, Forensic Chemistry Program, University of Mississippi
2002-2005	Distinguished Faculty Fellow, College of Liberal Arts, University of Mississippi

2003-present	Professor and Chair of Chemistry, University of Alabama at Birmingham
2003-present	Senior Scientist – Experimental Therapeutics Program, Comprehensive Cancer Center, University of Alabama at Birmingham
2003-present	Adjunct Professor, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham

FELLOWSHIPS, HONORS, AND AWARDS:

National Institutes of Health Research Service Award Traineeship (National Cancer Institute Postdoctoral Fellow) (1981-1984) in the laboratory of Professor Thomas R. Krugh, Department of Chemistry, University of Rochester

American Chemical Society Petroleum Research Award (1985-1988)

Cottrell Research Corporation Award (1985-1988)

Else U. Pardee Fellowship Award (1992-94)

Distinguished Faculty Fellow, College of Liberal Arts, University of Mississippi

PROFESSIONAL SOCIETIES:

American Chemical Society (1980-present)

American Association for Cancer Research (1988-present)

American Association for the Advancement of Science (1982-present)

Biophysical Society (1984-present)

Sigma Xi (1984-present)

Federation of American Society for Experimental Biology (1985-present)

American Society for Biochemistry and Molecular Biology (1986-present)

CONSULTANTSHIPS AND ADVISORY BOARDS:

Member, NIH Study Section – EBT (Enabling Bioanalytical and Biophysical Technologies) - (2008 – present)

Member, U.S. Army Breast Cancer Research Panel (USAMRMC) - (2001 - 2003)

Member, NSF, Molecular Biophysics Study Panel (1999 – 2003)

Member, Collaborative Research in Chemistry Study Panel, National Science Foundation (2002-2004)

Member, National Science Foundation Graduate Research Fellowship Review Panel (1999 – 2002)

Consultant, Shore Chan Bragalone, LLP

Chair, NIH Study Section - GGG-J (Genes, Genomes and Genetics) - (2007 - 2009)

Chair, NIH Study Section - IMST- (Genes, Genomes and Genetics) - (2009 - 2011)

Member, NSF GRFP Study Panel (Biochemistry and Molecular Biophys) – (2011-present)

RESEARCH INTERESTS:

DNA structural motifs as ligands for Toll-like receptors (TLRs)

DNA structure and stability as influenced by base sequence

Design and synthesis of novel topoisomerase I and II inhibitors as anticancer agents Mechanisms of action of anticancer agents

Structural and energetic properties of ligand-DNA interactions

Sequence and structurally selective interactions of DNA binding agents

UNIVERSITY COMMITTEE RESPONSIBILITIES:

(University of Mississippi)

Chairman, Institutional Biosafety Committee (Univ. Mississippi) (1989-2003)
Member, Graduate Council, University of Mississippi (1996-1998)
Alternate Member, Graduate Council, University of Mississippi (1998-2003)
Member, Environmental Safety Committee (Univ. Mississippi) (1989-2003)
Chairman, Biological Safety Sub-Committee (Univ. of Mississippi) 1991-2003)
Member, University Faculty Senate (Univ. Mississippi) (1996-2000)
Chairman, Faculty Governance Committee (1999-2000)
Member, Executive Committee of the Faculty Senate (Univ. Mississippi) (1999-2000)
Chairman, Information Technologies Committee (Univ. Mississippi) (1998-2000)
Member, University of Mississippi SACS Self-Study Committee on Administrative Processes (1997-2003)
Member, Tenure and Promotion Review Committee, (Univ. Mississippi) (1998-2003)
Member, Research Advisory Committee (Univ. Mississippi) (1996-2003)

(University of Alabama at Birmingham)

Member, Graduate Program Directors (UAB) (2003-present)
Member, Center for Computational and Structural Biology (2003-present)
Member, Transinstitutional Advisory Committee for the Center for Computational and Structural Biology (UAB) 2003-present)
Member, Executive Committee for Center for Computational and Structural Biology (2003-present)
Chair, Transinstitutional Advisory Committee for the Center for Computational and Structural Biology (2009-present)
Member, Institutional Advisory Committee for the UAB NMR Core Facility (2003-present)
Chair, UAB Comprehensive Cancer Center NMR Facility Advisory Committee (2003-present)
Member, UAB Research Advisory Group (2003-present)
Member, Executive Committee for UAB Research Advisory Group (2006-present)
Member, Drug Discovery Development Group, UAB Comprehensive Cancer Center, 2008-present
Member, Alabama Drug Discovery Alliance, 2008-present
Member, Search Committee for recruitment of UAB Dean of the Graduate School (2005-2006)
Chair, Search Committee for recruitment of UAB Chair of Department of Biology (2006-07)
Member, Executive Committee, Center for Lung Health (2009-present)
Member, UAB Center for Nanoscale Materials and Biointegration (CNMB)
Member, Internal Advisory Committee, UAB Center for Nanoscale Materials and Biointegration
Member, Chemical Safety Committee, UAB Occupational Health and Safety (2009-present)
Chair, Chemical Safety Committee, UAB Occupational Health and Safety (2009-present)
Member, Internal Advisory Committee, UAB Center for Structural Biology (2011-present)

PROFESSIONAL RESPONSIBILITIES:

Associate Editor

Anticancer Agents – Medicinal Chemistry, Bentham Publications, 2000-present

Journal Reviewer

Regular Reviewer, Biochemistry
Regular Reviewer, Cancer Research
Regular Reviewer, Biophysical Chemistry
Regular Reviewer, Biopolymers
Regular Reviewer, Nucleic Acids Research
Regular Reviewer, Proceedings of the National Academy of Science, USA
Regular Reviewer, Chemico-Biological Interactions
Regular Reviewer, Journal of Biological Chemistry
Regular Reviewer, Chemistry & Biology
Regular Reviewer, Journal of the American Chemical Society
Regular Reviewer, J. Natural Products

Granting Agency Reviewer

National Institutes of Health
National Science Foundation
American Cancer Society
American Chemical Society
Cottrell Research Corporation

TEACHING RESPONSIBILITIES:

Research Methods in Chemistry and Biochemistry	(Chem 201)
Biochemistry	(Chem 460)
Biophysical Chemistry	(Chem 463)
Advanced Biochemistry (I & II)	(Chem 461, 462)
Biochemistry Laboratory	(Chem 464)
Biochemical Techniques	(Chem 564)
Graduate Biochemistry (I & II)	(Chem 561, 562)
Physical Biochemistry	(Chem 563)
Principles & Applications of 1- & 2-D NMR Spectroscopy	(Chem 639, 739)

PROFESSIONAL SERVICE

Symposium Organizer

2012, Symposium entitled "Frontiers in Nucleic Chemistry", Southeast Regional meeting of the American Chemical Society, Raleigh, NC, Oct., 2012.

2011, Symposium entitled "Frontiers in Nucleic Chemistry", Southeast Regional meeting of the American Chemical Society, Richmond, VA, Oct. 26-29, 2011.

2010, Symposium entitled "Frontiers in Nucleic Chemistry", Southeast/Southwest Joint Regional meeting of the American Chemical Society, New Orleans, LA, Dec. 1-4, 2010.

2005, Symposium entitled "Frontiers in Nucleic Structure and Energetics", Southeast/Southwest Joint Regional meeting of the American Chemical Society, Memphis, Tennessee, Nov. 1-4, 2005.

2004, Symposium entitled "Nucleic Acids: Structural Motifs and Applications", Southeast Regional meeting of the American Chemical Society, Durham, North Carolina, Nov. 10-13, 2004.

2001, Symposium entitled "Topoisomerase Targeting Agents: Chemistry to Chemotherapy II", (National Cancer Institute funded), held on the University of Mississippi campus, August 25-28, 2001.

2000, Symposium entitled "Physical Chemistry of Nucleic Acids", Southeast – Southwest Regional Meeting of the American Chemical Society, New Orleans, Louisiana, December 6-8, 2000.

1998, Symposium entitled Sequence Selective Binding to DNA: Southwest Regional Meeting of the American Chemical Society, Baton Rouge, Louisiana, Nov. 1-3, 1998.

1998, Symposium entitled "Topoisomerase II Targeting Agents: Chemistry to Chemotherapy I (National Cancer Institute funded), held on the University of Mississippi campus, August 29-September 1, 1998.

1995, Southeastern DNA Symposium at the Southeast/Southwest Regional Meeting of the American Chemical Society, Memphis, Tennessee, Nov. 29-Dec. 1, 1995.

1994, Southeastern DNA Symposium at the Southeast Regional Meeting of the American Chemical Society, Birmingham, Alabama, October 7-9, 1994.

1990 Southeastern DNA Symposium, University of Mississippi, October 26-28, 1990.

PUBLICATIONS:

- Ketron, A.C., Denny, W.A., Graves, D.E. and Osheroff, N. (2012) "Amsacrine as a Topoisomerase II Poison: Importance of Drug-DNA Interactions" *Biochemistry* 51, 1730-1739.
- Aldred, K.J., McPherson, S.A., Wang, P., Kerns, R.J., Graves, D.E., Turnbough, C.L. and Osheroff, N. (2012) "Drug Interactions with *Bacillus anthracis* Topoisomerase IV: Biochemical Basis for Quinolone Action and Resistance" *Biochemistry*, 51(2) 370-381.
- Pitts, S., Jablonsky, M., Duca, M., Dauzonne, D., Monneret, C., Arimondo, P., Anklin, C., Graves, D.E., and Osheroff, N. (2011) "Contributions of the D-Ring to the Activity of Etoposide Against Human Topoisomerase II: Potential Interactions with DNA in the Ternary Enzyme-Drug-DNA Complex" *Biochemistry*, 50(22) 5058-5066.
- Gentry, A.C., Pitts, S.L., Jablonsky, M.J., Bailly, C., Graves, D.E., and Osheroff, N. (2011) "Interactions between the Etoposide Derivative F14512 and Human Type II Topoisomerases: Implications for the C4 Spermine Moiety in Promoting Enzyme-Mediated DNA Cleavage". *Biochemistry*, 50(15), 3240-3249.
- Hudson, J.S., Brooks, S.C., and Graves, D.E. (2009) "Interactions of Actinomycin D with Human Telomeric G-Quadruplex DNA" *Biochemistry* 48, 4440-4447.
- Ilvesaro, J.M., Merrell, M.A., Li, L., Wakchoure, S., Graves, D.E., Brooks, S., Rahko, E., Jukkola-Vuorinen, A., Vuopala, K.S., Harris, K.W., and Selander, K. (2008) "TLR9 mediates CpG-Oligonucleotide-Induced Cellular Invasion" *Molecular Cancer Research* 6, 1534-1543.
- Bender, R.P., Jablonsky, M.J., Shadid, M., Romaine, I., Dunlap, N., Anklin, C., Graves, D.E. and Osheroff, N. (2008) "Substituents on etoposide that interact with human topoisomerase II- α in the binary enzyme-drug complex: contributions to etoposide binding and activity". *Biochemistry* 47, 4501-4509.
- Bowling, J.J., Pennaka, H.K., Ivey, k., Wahyunono, S., Kelly, M., Schinazi, R.F., Valeriote, F.A., Graves, D.E., and Hamann, M.T. (2008) Antiviral and anticancer optimization studies of the DNA-binding marine natural product aaptamine". *Chem. Biol. Drug Discovery* 71, 205-215.
- Wilstermann, A.M., Bender, R.P., Godfrey, M., Choi, S., Anklin, C., Berkowitz, D.B., Osheroff, N., and Graves, D.E. (2007) "Topoisomerase II-Drug Interaction Domains: Identification of Substituents on Etoposide that Interact with the Enzyme". *Biochemistry* 46, 8217-8225.
- Graves, D.E. (2006) "Actinomycin D: Sixty Years of Progress in Characterizing a Sequence-Selective DNA Binding Agent" in series "Biomolecular Science - Sequence-Specific DNA Binding Agents" (ed. Michael Waring), Royal Society of Chemistry, Cambridge, pp. 109-129.
- Garbett, N.C., Hammond, N.B. and Graves, D.E. (2004) "Influence of the Amino Substituents in the Binding Energies of Ethidium with Nucleic Acids". *Biophysical Journal* 87, 3974-3981.
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- Carter, R.G., Graves, D.E., Gronemeyer, M.A. and Tschumper, G.S. (2002) "Synthesis of the ABC Ring System of Azaspiracid. 2. A Systematic Study into the Effect of C16 and C 17 Substitution on Bis-spirocyclization" *Organic Letters (Communication)*, 4(13), 2181-2184.
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Bailly, C., Graves, D.E., Ridge, G. and Waring, M.J. (1994) "Use of a Photoaffinity Derivative of Actinomycin to Investigate Shuffling Between Binding Sites on DNA." *Biochemistry*, 33, 8736-8745.

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Bailey, S.A., Graves, D.E., and Eftink, M.R. (1994) "Interactions of 7-Aminoactinomycin D with Single- and Double-stranded Oligonucleotides" in *Time-Resolved Laser Spectroscopy in Biochemistry IV* (ed. J. Lakowitz) Proc. SPIE, 462-468.

Bailey, S.A. and Graves, D.E. (1993) "Influence of DNA Base Sequence on the Binding Energetics of Actinomycin D", *Biochemistry* 32, 5881-5887.

Chaires, J.B., Priebe, W., Graves, D.E. and Burke, T.G. (1993) "Dissection of the Free Energy of Anthracycline Antibiotic Binding to DNA: Electrostatic Contributions" *Journal of the American Chemical Society* 115, 5360-5364.

Granzen, B., Graves, D.E., Baguley, B.C., Danks, M.K., and Beck, W.T. (1992) "Structure-Activity Studies of Amsacrine Analogs in Multidrug Resistant Human Leukemia Cell Lines Expressing Either Altered Topoisomerase II or P-Glycoprotein" *Oncology Research* 4, 489-496.

Gilbert, P.L., Graves, D.E. and Chaires, J.B. (1991) "Inhibition of the "B-Z" Transition in Poly(dGdC)-poly(dGdC) by Covalent Attachment of Ethidium: Equilibrium Studies" *Biochemistry* 30, 10925-10931.

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Wadkins, R. M. and Graves, D.E. (1991) "Interactions of Anilinoacridines with Nucleic Acids: Effects of Substituent Modifications on DNA Binding Properties" *Biochemistry* 30, 4278-4283.

Graves, D.E. and Wadkins, R.M. (1990) "Thermodynamic Studies of Amsacrine Antitumor Agents with Nucleic Acids" in *Molecular Basis of Specificity in Nucleic Acid-Drug Interactions* (ed. Bernard Pullman), Kluwer Academic Publishers, Dordrecht, Holland.

Wadkins, R. M. and Graves, D.E. (1989) "Thermodynamics of the Interactions of m-AMSA and o-AMSA with Nucleic Acids: Influence of Ionic Strength and DNA Base Composition", *Nucleic Acids Research* 17, 9933-9946.

Rill, R.L., Marsch, G.A., and Graves, D.E. (1989) "7-Azido Actinomycin D: A Photoaffinity Probe of the Sequence Specificity of DNA Binding by Actinomycin D" *J. Biomolecular Structure and Dynamics* 7, 591-605.

Krugh, T.R., Graves, D.E., and Stone, M.P., (1989) "Two-Dimensional NMR Studies on the Anthramycin-d(ATGCAT)₂ Adduct" *Biochemistry* 28, 9988-9994.

Eftink, M.R., Jia, Y-W., and Graves, D.E. (1989) "Intramolecular Fluorescence Quenching in an Acrylamide-Indole Adduct" *Photochem. Photobiol.* 49, 725-729.

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Elmore, R.H., Wadkins, R.M. and Graves, D.E. "Cooperative Binding of m-AMSA to Nucleic Acids" (1988) *Nucleic Acids Research* 16, 9707-9719.

Stone, M.P., Gopalakrishnan, S., Harris, T.M., and Graves, D.E. (1988) "Carcinogen-Nucleic Acid Interactions: Equilibrium Binding Studies of Aflatoxins B₁ and B₂ with DNA and the Oligodeoxyribonucleotide d(ATGCAT)₂" *J. Biomolecular Structure and Dynamics* 5, 1025-1043.

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Graves, D.E., Stone, M.P., and Krugh, T.R. (1985) "NMR Analysis of an Oligodeoxy- ribonucleotide-Drug Adduct." in *Molecular Basis of Cancer. Part B: Macromolecular Recognition, Chemotherapy, and Immunology. Interdisciplinary Discussion on the Basic and Applied Aspects of Cancer.* (ed. Rein, R.) Alan R. Liss, Inc., New York, New York.

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Graves, D.E. and Krugh, T.R. (1983) "Single-Cell Partition Analysis: A Direct Fluorescence Technique for Examining Drug-DNA Interactions." *Analytical Biochemistry* 134, 73-81.

Graves, D.E. and Krugh, T.R. (1983) "Adriamycin and Daunorubicin Bind in a Cooperative Manner to DNA." *Biochemistry* 22, 3941-3947.

Firth, W.J., III, Watkins, C.L., Graves, D.E., and Yielding, L.W. (1983) "Synthesis, Separation, and Characterization of Several Ethidium Analogs: Emphasis on Amino and Azido Substituents." *Journal of Heterocyclic Chemistry* 20, 759-765.

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Yielding, L.W., Graves, D.E., and Brown, B.R. (1979) "Covalent Binding of Ethidium Azide Analogs to Salmonella DNA In Vivo: Competition by Ethidium Bromide." *Biochemistry and Biophysics Research Communications* 87, 424-432.

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ABSTRACTS

Lanier, Kate and Graves, David E. Structural and thermodynamic analysis of a highly stable DNA hairpin. 63rd Southeast Regional Meeting of the American Chemical Society, Richmond, VA October 26-29, 2011.

Aldred, Katie J., McPherson, Sylvia A., Lindsey, R. H.; Wang, Pengfei; Kerns, Robert J., Graves, David E., Turnbough, Charles L. and Osheroff, Neil. "Quinolone Resistance in *Bacillus anthracis* Type II Topoisomerases" 63rd Southeast Regional Meeting of the American Chemical Society, Richmond, VA October 26-29, 2011.

Graves, David E.; Hudson, Jason S.; Ding, Lei; Ma, Jinbiao; Lewis, Edwin A. Recognition and binding of the human telomeric G-quadruplex by UP1 (Unwinding Protein 1) Joint 66th Southwest and 62nd Southeast Regional Meeting of the American Chemical Society, New Orleans, LA, United States, December 1-4 (2010).

Mitchell, Brandon A.; Hudson, Jason S.; Graves, David E. Characterization of a DNA binding peptide Joint 66th Southwest and 62nd Southeast Regional Meeting of the American Chemical Society, New Orleans, LA, United States, December 1-4 (2010).

Lanier, Katherine L.; Ottenfeld, Elise; Graves, David E. Structural analysis of a highly stable DNA Hairpin Joint 66th Southwest and 62nd Southeast Regional Meeting of the American Chemical Society, New Orleans, LA, United States, December 1-4 (2010).

Hudson, J.S., Brooks, S.C., and Graves, D.E. "Interactions of Actinomycin D with Human Telomeric G-Quadruplex DNA" 2nd International Meeting on Quadruplex DNA, Louisville, KY, April 18-21 (2009).

Brooks, S.C., Hudson, J.S., Selander, K., Graves, D.E. "Structure and Energetics of CpG Deoxyoligonucleotides that Induce TLR9 Mediated Cellular Invasion" 60th Southeastern Regional Meeting of the American Chemical Society, Nashville, TN, USA, November 12-15 (2008).

Mitchell, B., Bishop, G. R. and Graves, D.E. "Equilibrium of DNA Structural Motifs – DNA Aptamer and I-Motif versus Double-Stranded DNA" 60th Southeastern Regional Meeting of the American Chemical Society, Nashville, TN, USA, November 12-15 (2008)

Hudson, Jason; Brooks, Sonja; Graves, David. "The Binding of Actinomycin D to the G-Quadruplex DNA". Abstracts, 59th Southeast Regional Meeting of the American Chemical Society, Greenville, SC, United States, October 24-27 (2007).

Brooks, Sonja; Hudson, Jason; Graves, David E.. Characterization of the Binding of Ethidium Bromide to G-Quadruplex DNA. Abstracts, 59th Southeast Regional Meeting of the American Chemical Society, Greenville, SC, United States, October 24-27 (2007).

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Nadkarni, Dwayaja H.; Shinkre, Bidhan A.; Glover, Amanda L.; Raisch, Kevin P.; Graves, David E.; Velu, Sadanandan E. "Synthesis and biological evaluation of novel acridine-based topoisomerase I poisons." 58th Southeast Regional Meeting of the American Chemical Society, Augusta, GA. United States, November 1-4 (2006).

Hudson, Jason; Graves, David E.. "The binding energetics of actinomycin D to nonclassical sites in double-stranded DNA." Abstracts, 58th Southeast Regional Meeting of the American Chemical Society, Augusta, GA, United States, November 1-4 (2006).

Garbett, Nichola; Record, Jessica; Graves, David E.. "Energetics of Actinomycin D-DNA Interactions are Sequence-Specific. 57th Southeast/61st Southwest Joint Regional Meeting of the American Chemical Society, Memphis, TN, United States, November 1-4 (2005),

Record, Jessica; Graves, David. "Biophysical Characterization of Actinomycin D with Single-Strand DNA." 57th Southeast/61st Southwest Joint Regional Meeting of the American Chemical Society, Memphis, TN, United States, November 1-4 (2005).

Phillips, Jessica K.; Graves, David. "Hydrophobic contributions in ligand-DNA interactions." 229th ACS National Meeting, San Diego, CA, United States, March 13-17, 2005 (2005).

Hutchins, R.A. and Graves, D.E. (2003) "Interaction of Camptothecin with Nicked DNA Duplex", Southeastern Regional Meeting of the American Chemical Society, Nov. 16-18, 2003, Atlanta, GA.

Garbett, N. C. and Graves, D.E. (2003) "Thermodynamic Mechanisms of Actinomycin D-DNA Interactions are Sequence Specific", Southeastern Regional Meeting of the American Chemical Society, Nov. 16-18, 2003, Atlanta, GA.

Hutchins, R.A. and Graves, D.E. (2003) "Interaction of Camptothecin with Nicked DNA Duplex, 47th Biophysical Society Meeting, March 1-5, 2003, San Antonio, TX.

Godfrey, M., Wilstermann, A., Anklin, C., Osheroff, N. and Graves, D.E. (2003) "Characterization of the Binding Epitope of the Anticancer Agent, Etoposide, with its Cellular Target, Topoisomerase II Through Saturation Transfer Difference NMR", 47th Biophysical Society Meeting, San Antonio, TX, March 1-5, 2003.

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